ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

CyAn Quick User Guide

1. INSTRUMENT LAYOUT

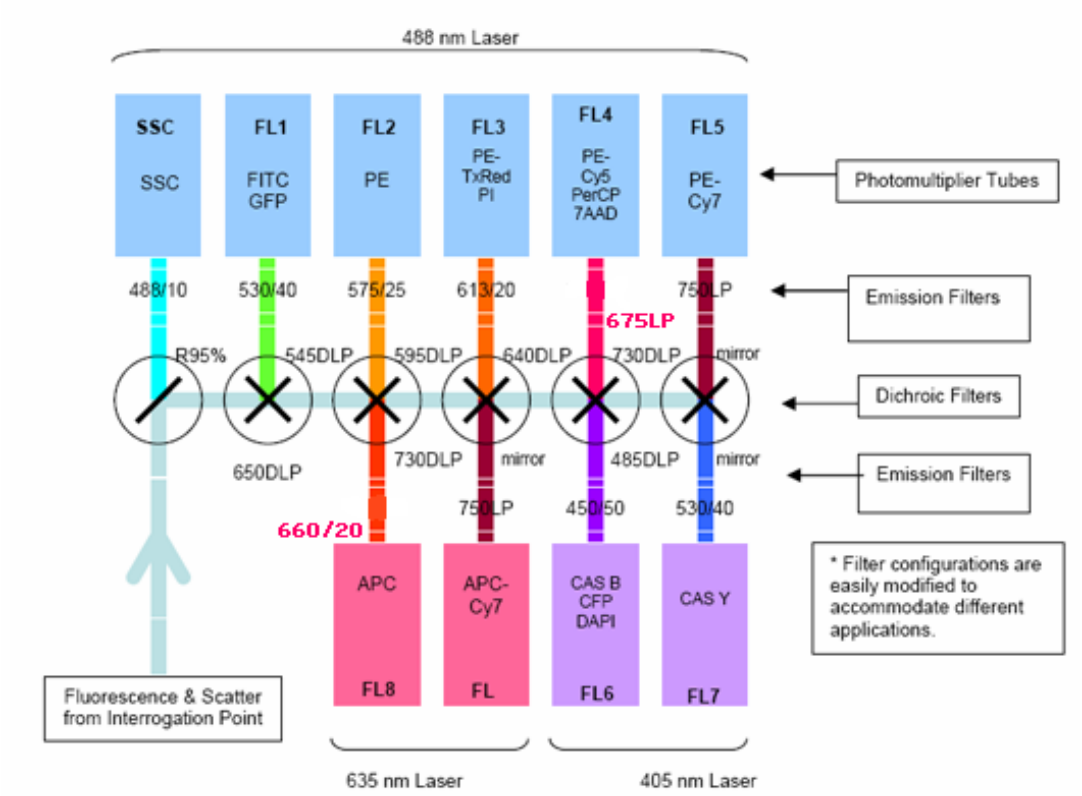


Figure. Optical Layout of a CyAn. Each group of detectors is indicated by colour. Blue detectors measure light from the blue laser (488nm), Red detectors measure light from the red laser (635nm), Violet detectors measure light from the violet laser (407nm).

Detector names (default) and commonly used dyes and fluorophores

- FITC (FL-1)	FITC, ALEXA 488, GFP, CFSE, YFP
- PE (FL-2)	PE, dsRED
- PE-TxRed (FL-3)	PI, PeAlexa610, PeTxRed
- PE-Cy5 (FL-4)	PeCy5, PeCy5.5, PerCP, 7AAD
- PE –Cy7 (FL-5)	PeCy7
- Violet 1 (FL-6)	Pacific Blue, DAPI, Hoechst for cell cycle, Live/Dead Violet
- Violet 2 (FL-7)	Pacific Orange
- APC (FL-8)	APC, Alexa 647
- APC-Cy7 (FL -9)	APC-Cy7, APC-Alexa750

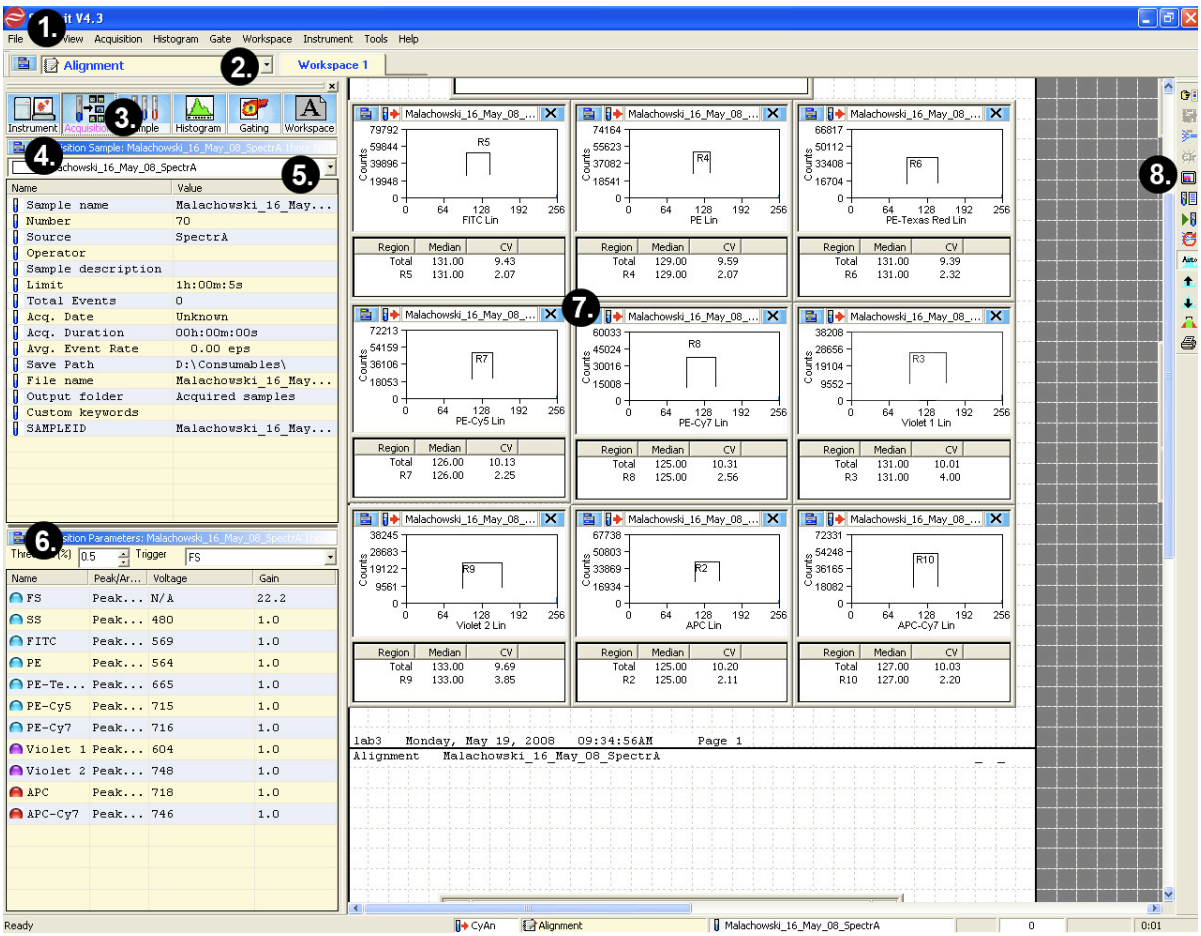
For others dyes and fluorophores please have a look at our web site, under Analyser – CyAn Analysers or ask the FCCF Staff.

From Monday to Friday, the FCCF staff runs a QC protocol on both CyAn's first thing in the morning. At the same time we are also taking care of emptying the waste and filling the sheath reservoir. When you arrive at the platform, the CyAns are turned on, the summit software is running and an empty database called "between users" is open. The machine is now ready to use!

2. SUMMIT OVERVIEW

Summit software is a Windows(R) based application that has a series of menus, hot keys, and buttons, which allow you to acquire data in FCS format. With Summit software you can monitor and control the instrument, define protocols, configure compensation settings and workspaces.

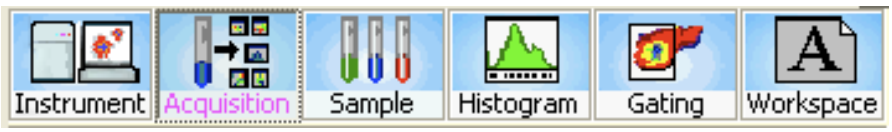
Summit Software Screen Overview



1. Summit software Main Menu
2. Protocol List
3. Summit Control Panel (to gain access to additional screens)
4. Additional Menu
5. Sample List
6. Additional Menu
7. Workspace (with blank histograms)
8. Toolbar Icons

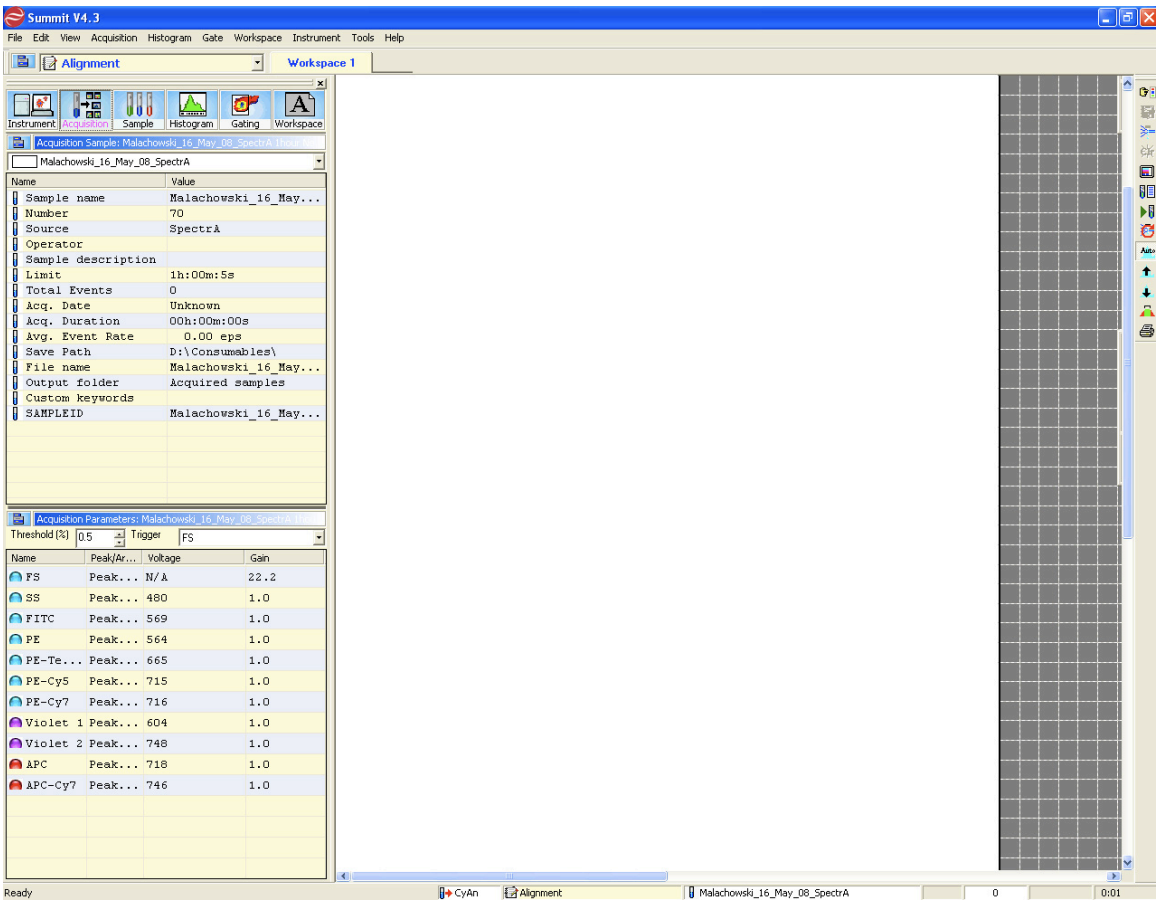
Summit Software Control Panel

Most of the operations in Summit software can be accessed through the Summit Software Control Panel. The panel is located on the left side of the screen and has a series of buttons across the top. You can select each of these buttons to get information related to a particular topic. Each tab contains submenus that have options specific to that menu.



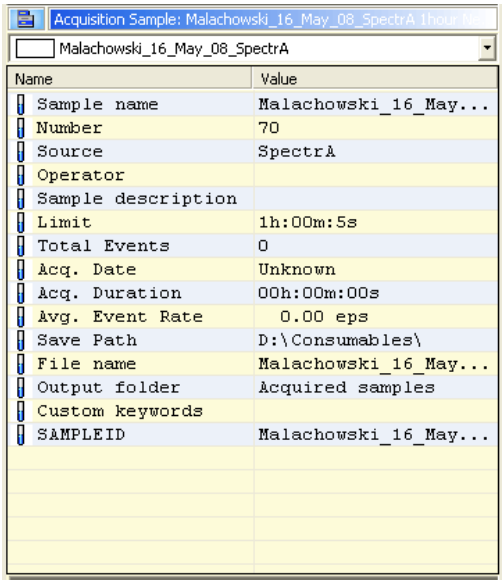
Acquisition Tab

The **Acquisition** tab allows you to set the threshold at which an event will be detected by the instrument, set the event triggering parameter, designate parameter names, specify the data types that will be collected, and set the voltage and gain to be applied to each parameter. From **Acquisition** tab you can also set up specific sample run information and view sample run statistics.

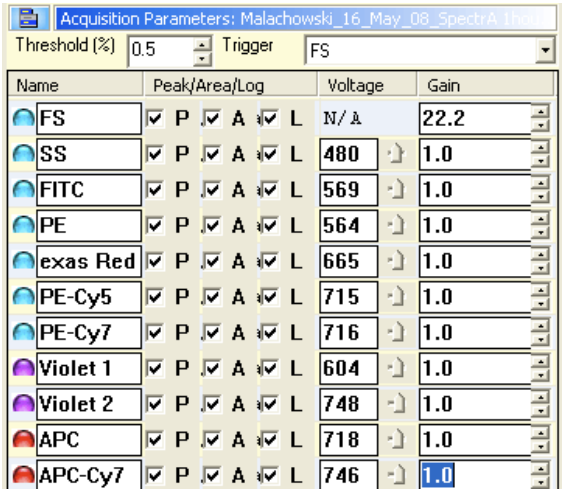


Acquisition Sample Panel

The Acquisition Sample Panel can be customized to display, and later saved, information specific to a sample run.

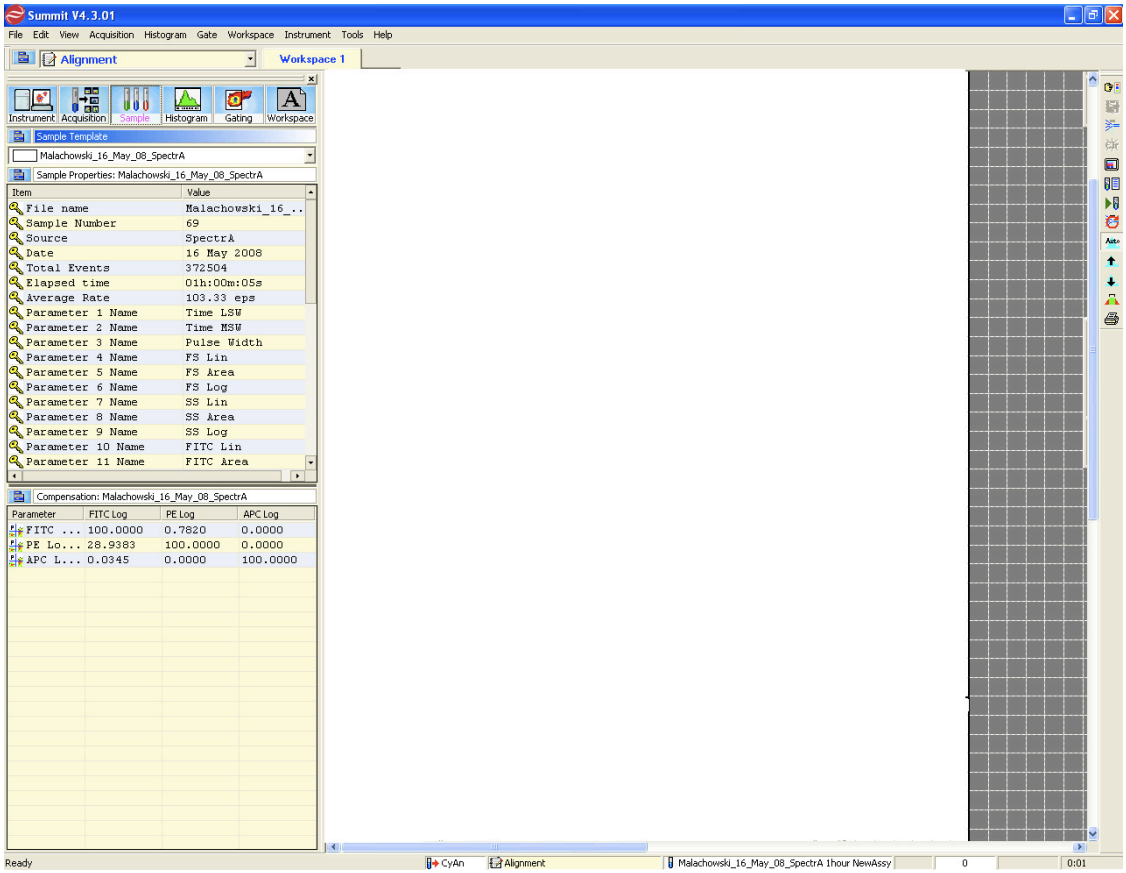


You can also edit all the acquisitions settings (parameters name, voltage, gain, trigger, threshold and data type parameter).



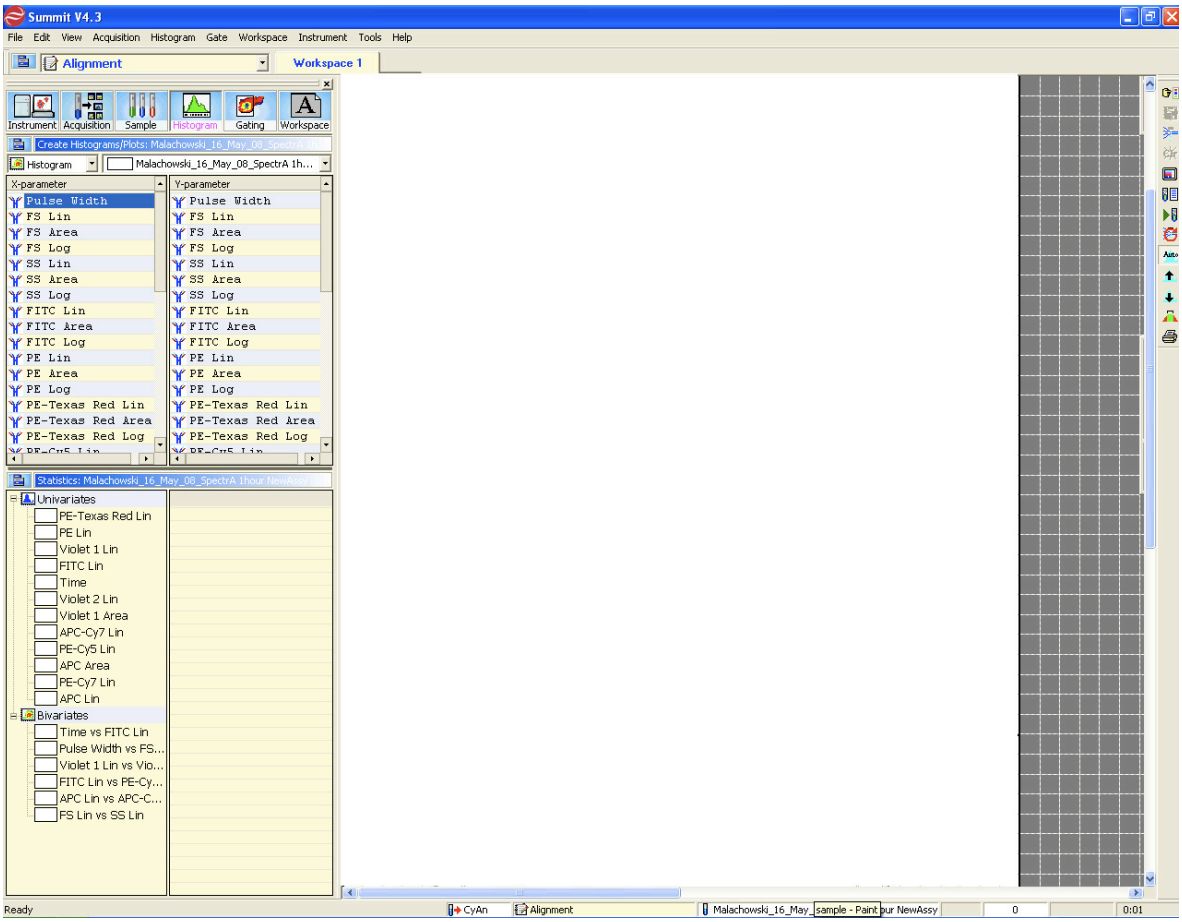
Sample Tab

Select this tab if you want to perform compensation. For a detailed description on how to do auto-compensation have a look at chapter 9.



Histogram Tab

Histograms and dot plots are created in the **Histogram** tab. The **Create Histograms** panel displays all of the parameters that are enabled in the **Acquisition** tab.



You must create histograms and dot plots in order to display the data you acquire. Prior to creating dot plots and histograms you must enable the parameters you would like to collect.

See chapter 7 on how to create graphs to view data

3. LOCATION AND TYPES OF DATA STORAGE

Personal folders

All data is stored in the **data storage drive D** on the Cyan's computer. There is a folder for each month, and each group or institute has a folder inside this folder. Each user has to create a personal folder inside their group folder.

Database

A database is essential to run Summit. It contains the **protocols** and **links** to all the files that are saved. Make a new database each time you begin an experiment (this minimises the problems with software bugs).

Protocol

Save your protocols into the folder **Settings for Everyone** in the 'data storage drive D'. A protocol is a collection of graphs and instrument settings that you use to run your experiment inside the database. These can be saved separately to the database to be re-used in subsequent experiments. Saving a protocol for future use is the easiest way to access settings and gates for next time. Saved protocols can only be re-used for experiments measuring exactly the same parameters – do not add or subtract parameters from the protocol after it has been saved.

Data Files

FCS 3.0 files saved by Summit contain all the measured signals that you saved for any given sample. They can be re-opened in Summit or opened in Flowjo for data analysis.

4. SAVING DATA

To set up Summit to save your data, make the necessary adjustments in the **Acquisition** tab.

Sample name

By double clicking on top row (sample name), you can adjust the rules that Summit uses to name your data file. A window appears with a 'rule string' listed along the bottom. Delete this and then re-create your own rule string by choosing the 'rules' that you wish to use.

Size of sample file

The size of your file is up to you. It is controlled in the **limit** row. Double click on the number of events to edit the limit. Leave **maximum events** at 10'000'000.

Hint: the size of your data file will be related to the proportion of cells you are interested in out of your total population.

Storage location

Use **Save Path** to specify the folder where your files will be saved. This folder should be inside a folder, which should be inside your personal folder.

Auto-save command

To ensure you remember to save your files, it is recommended to go to the drop down menu **Acquisition** and select **Auto-Save**. This makes the software request a 'save' each time you stop acquisition (ie each time you press F2).

Commanding Summit to save

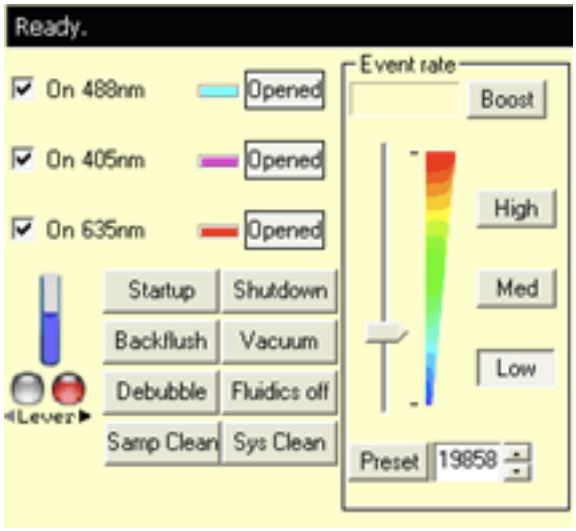
F3 is the shortcut key to save the data that has been passed through the machine. Press F3 at the end of any acquisition that you want to save. (This command is also in the acquisition drop down menu)

Files to save

As well as saving your experimental samples, save your unlabelled samples as well as any other controls (eg compensation controls) as these can help you to validate your data during data analysis.

5. START UP

1. Make a **new database** and save it to your folder
   a. Go to "File" menu and choose "Database" -> "New"
   b. Name the database and click Save
2. Select the "Instrument tab" (upper left) and click "Startup"



3. Place a ¾ full tube of "Decontamination" solution on the machine.
4. In the **Instrument** tab click **Sample Clean** (takes 60 seconds)
5. Remove the tube and an automatic "Backflush" occurs.
6. Repeat the steps above with a ¾ full tube of "Cleaning solution and then with a ¾ full tube of "H2O".
7. The Instrument is now ready to be set up for your experiment

6. CHOOSING AND NAMING PARAMETERS TO MEASURE

Before data can be acquired, data parameters must be enabled. Parameters are chosen and named in the 'Acquisition' tab.

Parameter activation

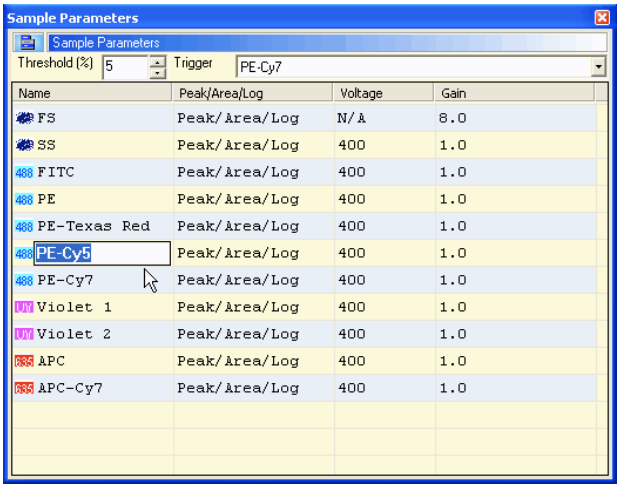
To activate a parameter, Double click in the **Peak/Area/Log** field. Select the check box that pertains to the data type that you want to acquire.

Peak	= FS & SS	Linear scale
Log	= Fluorescent antibody stains	Logarithmic Scale
Area	= Doublets discrimination	

To discriminate doublets, select Peak & Area for FS. To Cell Cycle users, please check settings with FCCF Staff.

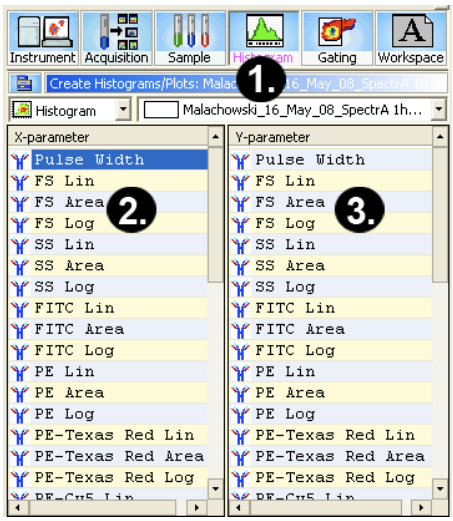
Parameter name

To change the name of a parameter, double-click the name of the parameter in the Name column, type a new name, and then press ENTER.



7. CREATING GRAPHS TO VIEW DATA

Graphs are created in the **Histogram** tab, top panel. Only fluorescent and scatter parameters chosen previously are available for graph creation.



1. Histogram Tab
2. X-axis Parameters
3. Y-axis Parameters

- To create a single parameter histogram, double-click on the X-axis parameter. The frame for the histogram will appear in the Workspace on the right of the screen.
- To create a dual parameter dot plot, click once on the X parameter and twice on the Y parameter. The newly created frame for the dot plot will appear in the Workspace.

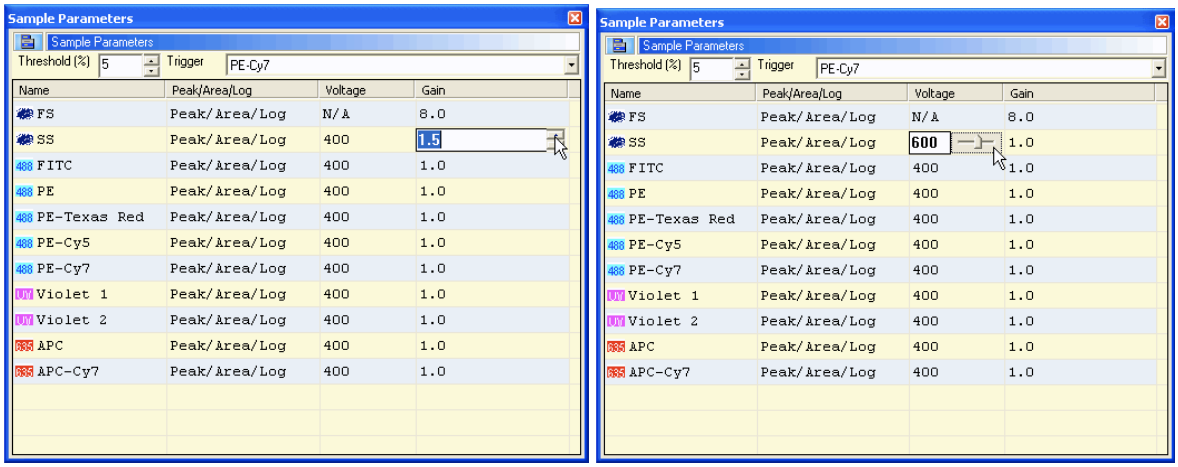
Suggestion: Create a histogram for each parameter to measure to use in order to optimise voltage settings in the next step.

8. OPTIMISING INSTRUMENT SETTINGS

Instrument settings need to be optimised for each experiment. An unlabelled sample is required to set the correct sensitivity for each parameter used in the experiment.

1. Place an unlabelled sample on the machine.
2. In the 'Instrument' tab, set the "Event rate" to "Low"
3. Press "F2" to start acquisition. Cells will begin to flow through the machine.
4. Adjust flow rate (up or down) until you have about 100 eps.
5. Looking at graphs, determine if cells are visible.
6. Optimise FS and SS parameters to get desired appearance in FS and SS plot.
7. Use 'Acquisition' tab and adjust FS gain up or down. Double click in the **Gain** field. Type a new value or click and drag the slider until the desired value is reached.
8. Use 'control Z' to refresh data display following settings adjustment

9. Select the **Acquisition** tab and adjust the SS voltage up or down. Double click the **Voltage** field. Type a new value or click and drag the slider until the desired value is reached.



10. Aim to place 'main population' in the central domain of FS/SS graph.

    Appearance of signals and optimisation of these parameters varies enormously between different types of samples (eg mesenchymal stem cells versus murine bone marrow versus polystyrene beads will all look very different from one another and have very different FS and SS settings)

11. Select FS as the Trigger. To change the trigger click on the arrow and select another parameter. The FS is most commonly used as the Trigger.
12. Check that the 'Theshold' setting is correct for your sample. Modify the threshold if necessary. Take care when adjusting the gain of the trigger as this also increases the threshold value.
13. Optimise the fluorescent parameters.
    a. For most fluorescent parameters select "Log"
    b. Right click on the histogram plot.
    c. Select "Adjust Voltage".
    d. Adjust voltage (click and drag the slider) until the peak is in the first log decade.
    e. Convention states that 'unlabelled' signal should be in "first log decade" (background signal).
14. Use 'control Z' to refresh data display.

Create a Gate

To create a gate, right click on graph and choose gating tool to draw gate. To apply gate to other plots, right click on gate itself and choose 'set gate'. Click once in each other plot until final plot, and then click twice in final plot. Gate is applied to all 'clicked' plots.

Flow Rate

For all standard applications, keep the CyAn Flow Rate below 22000 to ensure good quality data (stricter criteria apply when analysing cell cycle– talk to FCCF Staff).

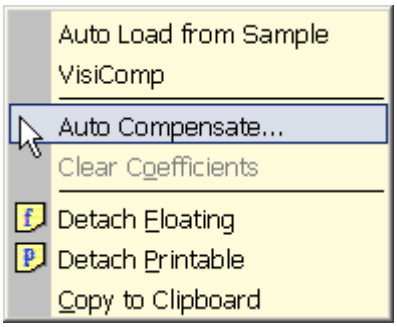
9. COMPENSATION

In many situations where you are measuring more than one fluorochrome in flow cytometry, you need to perform compensation to account for the 'overlap' of the measured fluorescent light between the different 'in use' parameters. The next steps can be done during the 'set up' phase, during data analysis of AFTER the experiment is complete. If you choose to the compensation during data analysis rather than during the 'set up' phase, it is ESSENTIAL that you save a copy of each of your 'single stain controls'.

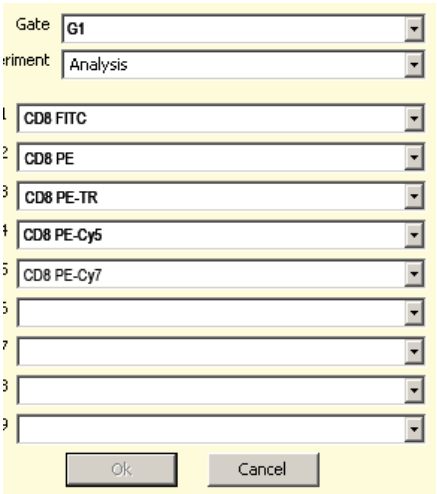
Auto-compensation:

Before you can perform automatic compensation, you have to first optimise the voltages for each of detector that you use and save a copy of each single stain control. To do the Auto-compensation:

1. Click the **Sample** tab.
2. In the **Sample Compensation** panel, click the small, blue icon in the upper-left corner and select **Auto Compensate** from the list.

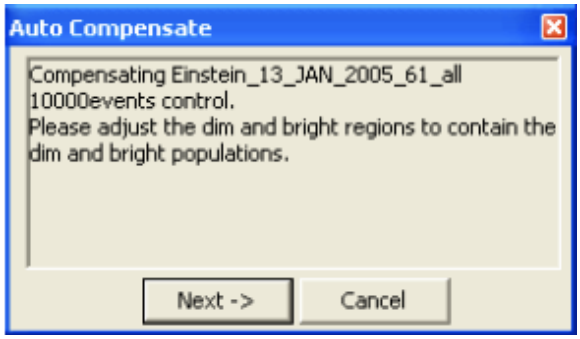


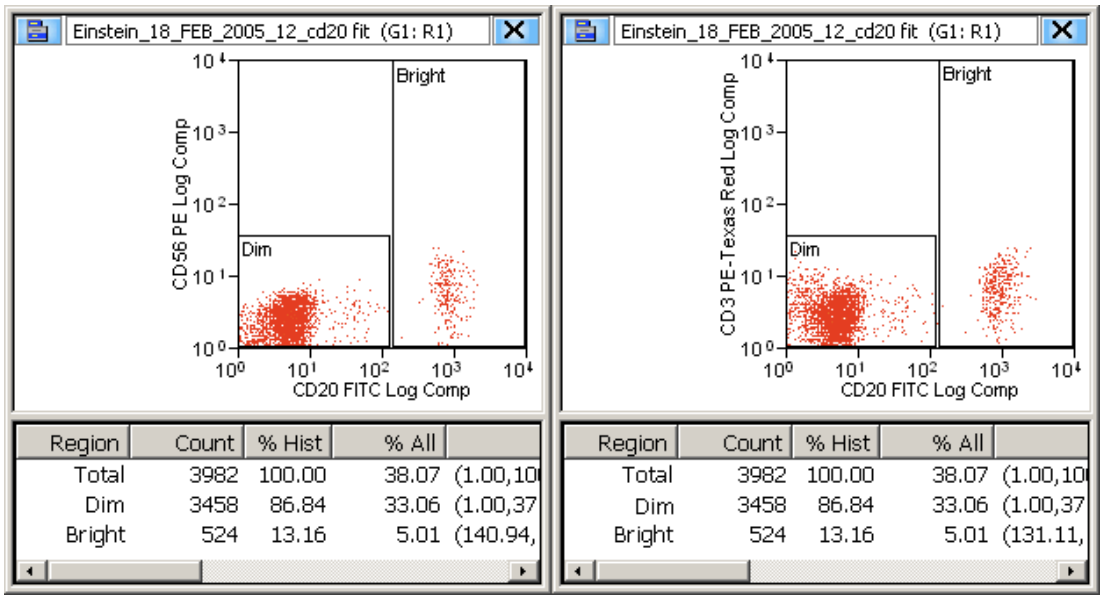
3. The **Auto Comp Sample** dialog box appears.



4. Select a gate from the Gate list, if applicable.

5. From the **Experiment** list, select the experiment folder that contains your control samples.
6. Allocated all of the single control samples to the appropriate channel. Click on the arrow to select the sample.
7. Click **OK**.
8. A new Workspace labelled **AutoComp** is created and the first set of dot plots is displayed. Each dot plot places the control parameter on the x-axis and a parameter to compensate against on the y-axis. Default auto compensation **Dim** and **Bright** regions are displayed and, if a gate was selected, it is applied to each dot plot.
9. At the same time the **Auto Compensate** wizard appears.

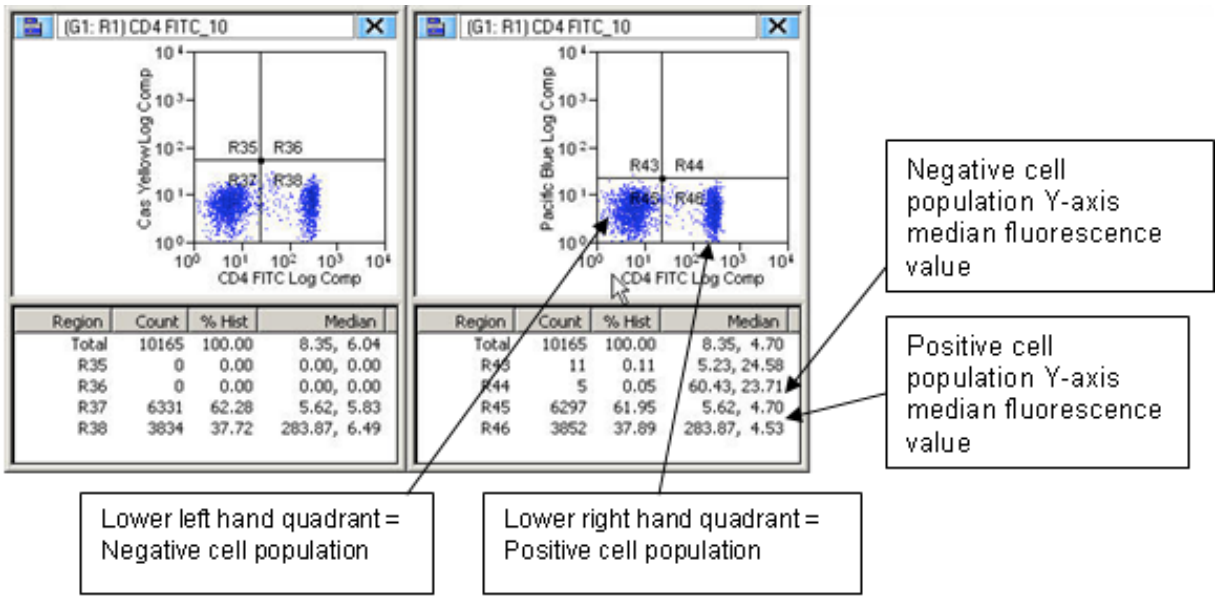




10. Examine the *% Hist* statistics for each histogram. If either the **Dim** or **Bright** region contains less than 5% of the data for the dot plot, click-and-drag the region until greater than 5% of the data appears in both the **Dim** and **Bright** regions.
11. When all regions on all plots contain greater than 5% of the data, click **Next** on the **Auto Compensate** dialog box and the next set of dot plots will appear.
12. Repeat step 10 until all single-control samples have been compensated. When auto-compensation is complete, the compensation matrix contains the appropriate values and the **AutoComp** workspace is removed. Compensation matrix can be inspected in bottom panel of "SAMPLE Tab"

Manual compensation (Advanced users only!):

1. Click the menu icon in the top left of the actual graphs and choose **Compensate**.
2. Adjust scroll bars to compensate samples appear. Adjust the spill over percentage so that the Y-axis median fluorescence value for the positive population (lower right hand quadrant) is equivalent to that seen with the negative population (lower left hand quadrant).



3. It is also possible to enter numbers by hand into compensation matrix under **Sample** tab.

10. CLEAN UP/SHUT DOWN ROUTINE

Once all data has been saved, it is time to shut down the instrument.

We have two shutdown procedures:

A. **Monday till Friday, from 8:00 am to 5:00 pm**
B. **After 5:00 pm or in the weekends**

A. Monday till Friday, from 8:00 am to 5:00 pm

1. Place a ¾ full tube of "Decontamination" solution on the machine.
2. In the **Instrument** tab click Sample Clean (takes 60 seconds)
3. Remove the tube and an automatic "Backflush" occurs.
4. Repeat the steps above with a ¾ full tube of "Cleaning solution" and then with a ¾ full tube of "H2O".
5. Go to File menu, and select "Database" and then "Load", select the "Between Users Database" on the desktop
6. In the **Instrument** tab click "Fluidics off"
7. Place a ¾ full tube of "H2O"; remove the tube and an automatic "Backflush" occurs.
8. The fluidic is now OFF and you can now leave the machine like this.

B. After 5:00 pm or in the weekends

1. Place a ¾ full tube of "Decontamination" solution on the machine.
2. In the **Instrument Tab** click Sample Clean (takes 60 seconds)
3. Remove the tube and an automatic "Backflush" occurs.
4. Repeat the steps above with a ¾ full tube of "Cleaning" solution and then with a ¾ full tube of "H2O".
5. After running the water, go to File, select "Database" and then "Load", select the "Between Users Database" on the desktop

Check the reservations on the SV-INTRANET if there's another user after you :

1. In the "Instrument Tab" click "Fluidics off"
2. Place a ¾ full tube of "H2O"; remove the tube and an automatic "Backflush" occurs.
3. The fluidic is now OFF and you can leave the machine like this.

If there's no user after you:

6. In the **Instrument** tab choose, "Shutdown".
7. Place a ¾ full tube of "Cleaning solution" on the machine.
8. In the **Instrument** tab choose, sample clean.
9. Place a ¾ full tube of "H2O" on the machine.
10. In the **Instrument** tab choose, sample clean.
11. Leave the machine with a tube of H2O on the sample probe.
12. Turn OFF the computer. Do not turn off the CyAn.

If you cancel your reservation after 5pm and you are supposed to be the last user of the day, you are responsible for shutting down the machine!

11. DATA EXPORT

Data analysis of the saved files is **always** performed on another computers. To get saved files to your own computer, you can use a USB key, burn a CD or use the network to connect to your "share-lab" folder.

Data is removed from the computer at the end of every month by the FCCF staff. Each user is responsible for backing up his data. Please backup your data right after you finished acquiring your samples.

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